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Effects of dimethoate, an organophosphate insecticide, on photosynthesis of five selected phytoplankton species

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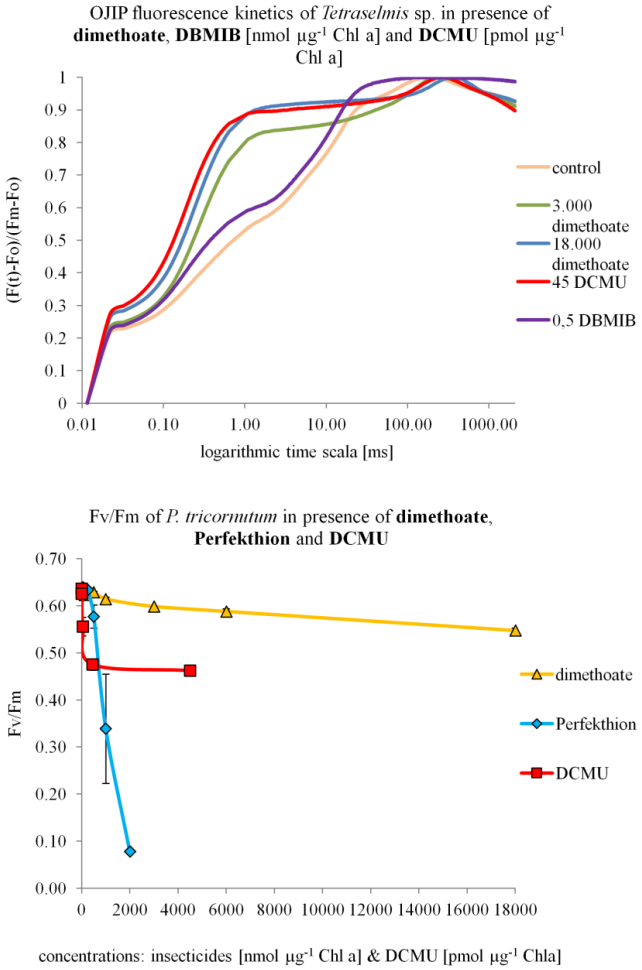
Graphical abstract



Abstract

Several studies in the late eighties have shown that the insecticide dimethoate is not only toxic to insects due to the inhibition of the acetylcholinesterase, but also to phototrophs by the inhibition of photosynthesis. Although the use of dimethoate and also its commercial formulations are not any longer permitted in the EU, it is still under application mostly in private hands or small farms in the Mediterranean area and in legal use in many non-EU countries. The mode of action in the aquatic environment is not clear until now. In the present study we have extended the aquatic toxicity test via photo-synthetic oxygen production by the analysis of Chl a - *in vivo* fluorescence and xanthophyll cycle pigments, which indicate the capacity to resist against photoinhibition. The study presents a data set including three different green algae and two diatoms to test the variety of responses from the major algal taxa in the aquatic environment. The data show that dimethoate as well as the commercial product Perfekthion inhibits PSII in a similar manner as DCMU (3-(3, 4-Dichlorophenyl)-1, 1-dimethylurea), a PSII inhibitor that decreases the photosynthetic oxygen evolution rate. The formulated product shows a higher toxicity than the pure chemical compound, which indicates that the formulation cocktail increases stability/permeability as key elements controlling toxicity, leading probably to photoinhibition.

Keywords: Dimethoate, insecticide, Perfekthion, phytoplankton, photosynthesis, oxygen rate, fluorescence, photochemical efficiency, non-photochemical quenching, xanthophylls

1. Introduction

Phytoplankton play a key role in aquatic ecosystems as primary producers of the aquatic food chain. The planktonic algae enhance the rapid nutrient recycling as well as the removal of soluble organic substances. Although the phytoplankton biomass in the world's oceans amounts to not more than 1-2% of the total global plant carbon, it can fix up to almost 50% of the photosynthetic carbon every year (Falkowski *et al.*, 1998; Field *et al.*, 1998).

In recent years, new challenges in environmental management have arisen with a variety of environmental pollutants establishing their presence (Musmarra *et al.*, 2019). Among these, pesticides are increasingly used in the modern society, posing serious environmental hazards. They may enter water ecosystems through spray, drift, leaching, runoff, or accidental spills (Van der Werf, 1996), affecting non-target organisms such as phytoplankton (Kungolos *et al.*, 2009). The use of organophosphate (OP) insecticides increased drastically in the past decades because of their low persistence in the environment (Epa, 2006; Petsas *et al.*, 2007; Vagi *et al.*, 2005) and high

efficiency against pests. Dimethoate is an important OP insecticide with foliar application used to kill insects by contact and stomach action (Tomlin, 1997). It is an acetyl cholinesterase inhibitor that has been used in the Greek agriculture primary against the fruit fly (*Bactrocera oleae*, *Dacus oleae*), which lays its eggs into the olive fruit. However, besides its efficiency in killing insects, several studies have shown that photosynthesis is the main target of dimethoate in phytoplankton species (e.g. Wong and Chang, 1988; Kobbia *et al.*, 1991; Perona *et al.*, 1991a; Mohapatra & Schiewer, 1998; Chen *et al.*, 2007; Moermond *et al.*, 2008). The same is also true for Rogor, which has been the commercial product of dimethoate used in agriculture and which after some years was renamed to Perfekthion, containing 37% dimethoate as active ingredient (a.i.) (Adhikary, 1989; Panda, 1998; Surekha, 1999; Mohapatra & Schiewer, 2000; Jena *et al.*, 2012).

According to guidelines from the Federal Office of Consumer Protection and Food Safety of Germany (BVL, 2019) and the European Food and Safety Authority (EFSA, 2022), the EU commission prohibited the use of plant protection products containing dimethoate after the 30th of June 2020 (EU, 2019a,b). However, these products are still in use in many other non-EU countries. Furthermore, the use of non-registered plant protection products is still significant in many rural parts of Greece.

Little is known about the precise target of dimethoate within the photosynthetic apparatus of phytoplankton cells. Different effects have been described, such as inhibition of the electron transport between photosystem I (PSI) and photosystem II (PSII) of *Nostoc* cells (Chen *et al.*, 2007), increase of PSII fluorescence (Mohapatra *et al.*, 1997), and inhibition of PSII activity in *Synechocystis* sp. (Mohapatra *et al.*, 1996). For Rogor, it has been reported that it inhibits the PSII-PSI electron-flow in *Chlorella vulgaris* (Jena *et al.*, 2012) and in *Synechocystis* sp. (Mohapatra, 2000).

The measurement of photosynthetic oxygen production is routinely performed by using a Clark electrode. While this measurement allows quantification of the total efficiency of the photosynthetic light reaction, fluorescence-based measurements as OJIP transients and NPQ measurements enable mechanistic insights in single steps of the photosynthetic electron transport chain (ETC). The OJIP fluorescence reflects the time course of energy transfer from PSII to PSI. O, J, I and P fluorescence levels are induced by different redox states of the ETC (Stirbet & Govindjee, 2011). At O-level, the PSII reaction centers are open, i.e. Q_A is oxidized, hence fluorescence is minimal (F_o). At P-level, all PSII reaction centers are in a closed state and fluorescence is maximal (F_m). The J- and I-level are intermediate levels, whereas the J-level is a reflection of the exchange of a reduced plastoquinone-molecule (Pq) for an oxidized Pq at the Q_B-site, while the I-level reflects the rate limitation imposed by the re-oxidation of plastoquinol molecules at the cytochrome (cyt) b_6f-complex (Schansker *et al.*, 2005). The variable fluorescence is the difference between F_m and F_o. The maximum photochemical efficiency F_v/F_m describes the maximum efficiency of a photon trapped in the antenna to induce photochemistry at PSII reaction centers (Schreiber *et al.*, 1994).

Plants and algae employ a mechanism to protect themselves from the adverse effects of high light (HL) intensity, where light energy absorption exceeds the capacity for light utilization in photosynthesis, by dissipating the excessive energy as heat (NPQ) (Müller *et al.*, 2001; Horton *et al.*, 2005; Baker, 2008). HL conditions increase the transthylakoidal proton gradient (low pH in the lumen), which activates the de-epoxidizing enzymes of the xanthophyll cycles. By interacting with antenna proteins, the newly formed pigments of the xanthophyll cycles induce a conversion of excessively absorbed light energy into heat. Two major photoprotective xanthophyll cycles are known: the violaxanthin cycle (Vx cycle), primarily of the green algae (Chlorophyceae) and land plants (Hager, 1980), and the diadinoxanthin (Dd) cycle of the diatoms (Bacillariophyceae). Under HL conditions violaxanthin (Vx) is converted via antheraxanthin (Ax) to zeaxanthin (Zx) in the Vx cycle (Sapozhnikov *et al.*, 1957; Yamamoto *et al.*, 1962) and diadinoxanthin (Dd) to diatoxanthin (Dt) in the Dd cycle (Hager & Stransky, 1970). The de-epoxidation state (DES) of each cycle can be determined by measuring the respective xanthophyll pigment concentrations by HPLC.

NPQ can be composed of the high-energy state (qE), the state transition (qT) and the photoinhibitory (qI) quenchings (Müller *et al.*, 2001; Goss & Lepetit, 2015). qI is either caused by the inactivation or damage of PSII reaction centers or by stable quenching in the PSII antenna. In diatoms, qT does not exist and its NPQ mainly relies on qE, a quenching mechanism, which is controlled by the build-up of a transthylakoidal proton gradient (ΔpH), the xanthophyll cycle and the presence of specific polypeptides of the light-harvesting complex (LHC) antenna, named Lhcx (Lavaud, 2007; Bailleul *et al.*, 2010; Buck *et al.*, 2019). The requirements of NPQ in green algae (violaxanthin cycle, proton gradient and LHCII aggregation) are in principle comparable to those of higher plants (Goss & Lepetit, 2015), but instead of PsbS, green algae rely on LhcSR proteins (related to Lhcx) (Bassi Dall'Osto, 2021) and the importance of the xanthophyll cycle for qE is species dependent (Quaas *et al.*, 2015).

The aim of the study was to investigate the impact of dimethoate on the photosynthesis of phytoplankton species from different taxonomical classes. Furthermore, this research may improve our understanding of the connection between the ETC, the xanthophyll pigment de-epoxidation and NPQ and enrich our knowledge on the ecotoxicology of OPs.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade and purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), unless otherwise stated. Dimethoate (2-dimethoxyphosphinothioylsulfanyl-*N*-methylacetamide, CAS No. 60-51-5) with a purity ≤ 100% was used in all

experiments by Clark electrode, PAM and HPLC, while the PSII inhibitor DCMU (3-(3,4-Dichlorophenyl)-1,1-dimethylurea, CAS-No. 330-54-1) was used as a positive control. The effect of 3 µmol dimethoate μg^{-1} Chl a and 45 pmol DCMU μg^{-1} Chl a on photosynthesis of the tested phytoplankton species was compared in all experiments, as a similar effect of both pesticides was observed in the OJIP fluorescence kinetics of the tested species (except in *Tetraselmis* sp.). DBMIB (2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone, CAS-No. 29096-93-3), an inhibitor of cyt b_6f complex, was used as a positive control in the OJIP fluorescence experiments. Perfekthion (BASF SE company, Ludwigshafen, Germany), the commercial formulation of dimethoate, is a blue emulsifiable liquid that contains 37,2% (w/w) dimethoate as active ingredient (a.i.), 43.5-48% cyclohexanone (CAS-No. 108-94-1), 4.2-5.2% solvent naphtha (CAS-No. 64742-94-5) and 4.2-5.2% acetic anhydride (CAS-No. 108-24-7). All reagent concentrations are referred to final concentrations in the phytoplankton samples and are dissolved in ethanol. The control of each reagent was 100% ethanol.

2.2. Phytoplankton species and culture media

The three Chlorophyceaen species *Chlamydomonas reinhardtii, Dunaliella tertiolecta* and *Tetraselmis* sp., and the two Bacillariophyceaen species *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* were grown in 250 ml flasks (20°C), in a day/night rhythm of 16/8 h, with a white light intensity of 15-45 µE m^{-2} s^{-1} (LL), on shakers (120-130 rpm). The cells were counted per Coulter Counter instrument (Multisizer 3, Beckman, Indianapolis, USA), as only phytoplankton cells in exponential growing phase were used in the experiments.

The type of the culture media and the origin of the phytoplankton species are described in Table 1. The pH of all used culture media was adjusted with hydrochloric acid to 7.0. In *f2* medium the organic compound Tris (pH 8) was added as a buffer.

$f2_{Si/Se}$ medium was prepared for *T. pseudonana* using *f2* medium according to Guillard and Ryther (1962) and Guillard (1975). 4.5 ml *f2* nutrient solution, 1 ml of a 0.82 M Na_2SiO_3 and 11.6 µM H_2SeO_3 solution were added to 1 L artificial seawater (ASW) via a sterilised filter syringe.

The recipe for *C. reinhardtii* culture medium was obtained from Pringsheim and Koch (1964).

2.3. Cell lysis and chlorophyll a determination

Chlorophyll a (Chl a) of the algal culture was determined in order to use comparable pesticide concentrations (μg^{-1} Chl a) in the experiments. For this purpose, 3 ml of each phytoplankton species was centrifuged for 4 min at 4000 x g and 20°C (Beckman Coulter, Allegra 25R Centrifuge, Krefeld, Germany). The supernatant was discarded, methanol (100 µl) and glass beads were placed on the cell pellet and vortexed (Vortex-Genie 2, Scientific Industries, New York, USA). Acetone (900 µl) was added and the mixture was vortexed, then centrifuged for 1 min at 4000 x g and 20°C. The absorbance of the supernatant (2 replicates) was measured (Ultrospec 2100 pro, UV/Visible Spectrophotometer, Biochrom, Cambridge, England). The Chl a concentration of the tested Bacillariophyceaen and Chlorophyceaen species was calculated according to the formula of Jeffrey und Humphrey (1975), as adapted by using the measurements of Ritchie (2006) (formulas 1 - 2).

Bacillariophyceae:

$$Chl\ a\ =\ 11.49\ x\ \left(E_{664}-E_{750}\right)\ -\ 0.45\ x\ \left(E_{630}-E_{750}\right)\left[\mu g\ ml^{-1}\right] \quad (1)$$

Chlorophyceae:

$$Chl\ a\ =\ 11.87\ x\ \left(E_{664}-E_{750}\right)\ -\ 1.79\ x\ \left(E_{647}-E_{750}\right)\left[\mu g\ ml^{-1}\right] \quad (2)$$

Chl a: chlorophyll a; E_x: absorbance at x nm wavelength

2.4. Oxygen evolution rate measurements by clark electrode

The photosynthetic oxygen evolution rate and the respiration of the tested phytoplankton species was tested with the Clark electrode in presence of 0.125-18 µmol μg^{-1} Chl a dimethoate and 12.5-2000 nmol μg^{-1} Chl a Perfekthion. The concentration of the phytoplankton species was adjusted to 2 µg Chl a ml^{-1} and after acclimatisation (1 h) to low light (LL), 2 ml of the sample was placed into the reaction chamber of a Clark electrode (Hansatech Instruments, Oxy-Lab, Helmut Saur, Laborbedarf, Reutlingen, Germany). Algal respiration was measured as negative oxygen evolution rate under dark conditions (4 minutes). Immediately afterwards, under light condition (150 µE m^{-2} s^{-1} light intensity), the net photosynthetic oxygen evolution rate of the algae was calculated, first in absence (4-6 minutes illumination) and then in presence of dimethoate/Perfekthion (for another 4-6 minutes). 4.5-4500 pmol DCMU μg^{-1} Chl a was used as a positive control. Under the following dark condition (4 minutes), the algal respiration was measured in presence of the insecticide.

2.5. Fluorescence measurements by PAM instrument

The fluorescence measurements were performed by an Aqua Pen instrument (Aqua Pen-C, AP-C 100, Photon Systems Instruments, Drasov, Czech Republic). Each phytoplankton sample was adjusted to 1.0 µg Chl a ml^{-1} and acclimatised (1 h) to LL. The insecticide was added to 1 ml of each algae and dark acclimated (10 minutes). Afterwards, it was pipetted into a cuvette and OJIP fluorescence and NPQ were measured under blue excitation light (450 nm).

The final concentrations of dimethoate, Perfekthion, DCMU, and DBMIB of the samples are listed in Table 2, according to the tested phytoplankton species and the experiments.

Specifically, for OJIP fluorescence measurements, the intensity of the measuring light was 3.3 nE m^{-2} s^{-1} and of the saturating flash 2100 µE m^{-2} s^{-1}.

Table 1. Phytoplankton species used in the study with their respective origin, culture medium, salinity and pH

species	origin	culture medium	salinity	source
Tetraselmis sp.	SAG (Culture Collection of Algae, Goettingen University, Germany)	*f2*	35‰	Guillard & Ryther (1962) and Guillard (1975)
D. tertiolecta	SAG 183.80	*f2*	35‰	Guillard & Ryther (1962) and Guillard (1975)
C. reinhardtii	SAG 11-32a	*Chlamydo-monas* medium	0‰ (bidistilled water)	Pringsheim & Koch (1964)
P. tricornutum	CCAP 1052/6 (UTEX 646, SAG 1090-6), Finland	*f2*	35‰	Guillard & Ryther (1962) and Guillard (1975)
T. pseudonana	CCMP 1335 Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor	$f2_{Si/Se}$	35‰	Guillard & Ryther (1962) and Guillard (1975)

Table 2. Final dimethoate, Perfekthion, DCMU, and DBMIB concentrations of the fluorescence experiments

measurements	phytoplankton species	concentrations [nmol µg^{-1} Chl a]			
		dimethoate	Perfekthion	DCMU	DBMIB
OJIP	3 Chlorophyceaen (i.e. *Chlamydomonas reinhardtii*, *Dunaliella tertiolecta* and *Tetraselmis* sp.) & 2 Bacillariophyceaen (i.e. *Phaeodactylum tricornutum* and *Thalassiosira pseudonana)*	125-18000	12.5-2000	0.045	-
	Tetraselmis sp.				0.5
F_v/F_m	3 Chlorophyceaen & 2 Bacillariophyceaen	125-18000	12.5-2000	0.0045-4.5	-
NPQ_L	3 Chlorophyceaen & 2 Bacillariophyceaen	125-18000	25-2000	0.045	-
NPQ_{DR}	3 Chlorophyceaen & 2 Bacillariophyceaen	3000	500	0.045	-

Table 3. NPQ protocol with duration and saturated light pulse details for the NPQ measurements with the Aqua Pen instrument (PSI, online)

	Phase	Duration	Nr. of pulses	1st pulse	Pulse interval
NPQ	Light (NPQ_L)	200 s	10	10 s	20 s
	Dark Recovery (NPQ_{DR})	390 s	7	20 s	60 s

For NPQ measurements, dark-acclimated algae were exposed to actinic irradiance (700 µE m^{-2} s^{-1}, 200 s) to elicit a transient Kautsky effect. Moreover, a sequence of saturating flashes was applied on top of the actinic light to probe NPQ in the light adapted state (NPQ_L) (for details see Table 3). NPQ_L was automatically calculated using the Stern-Volmer parameter (formula 3):

$$NPQ_L = F_m / F_{m'} - 1 \quad (3)$$

F_m: maximum fluorescence in the dark; $F_{m'}$: maximum fluorescence in the light

After exposure to continuous illumination, the relaxation of NPQ was determined by means of saturating light pulses applied in the dark (390 s) (Table 3). NPQ dark recovery (NPQ_{DR}) of dimethoate/Perfekthion- and DCMU-treated phytoplankton samples (Table 2) was automatically calculated and compared to the last measured NPQ_L of the control in order to examine the maximum recovery condition of each phytoplankton species.

2.6. Xanthophyll pigment measurements by HPLC

Specific xanthophyll pigments of the phytoplankton samples were quantified by reversed phase HPLC (VWR-Hitachi LaChrom Elite) to determine the DES of the xanthophyll cycle. In order to induce the de-epoxidation, the samples in the Clark electrode were illuminated with 500 µE m^{-2} s^{-1} light intensity. 0.125-3 µmol dimethoate µg^{-1} Chl a, 0.5 µmol Perfekthion µg^{-1} Chl a and 0.045-4.5 nmol DCMU µg^{-1} Chl a were added to each sample at the beginning of the measurements under dark conditions (4 minutes). After 10 minutes of illumination, samples were filtered through 1.2 µm Membrane Isopore Polycarbonate filters (Millipore, USA), the filters were flash-frozen in liquid nitrogen and stored at -80°C until further HPLC analysis.

Pigment extraction and HPLC separation were performed according to the protocol of Jakob *et al.* (1998). The pigments of the filtered cells were extracted with a mixture (700 µl) of 81% methanol / 9% 0.2 M ammonium acetate / 10% ethyl acetate and after adding a minor amount of glass beads, vortexed and centrifuged (Eppendorf Centrifuge 5415 D, Hamburg, Germany) at 13200 rpm for 2.5 min. 400 µl of the supernatant from each sample tube was injected onto a calibrated HPLC system equipped with a 10°C-cooled autosampler (L-2200), a photodiode array (model L-2455) and a Nucleosil 120-5 C18 column (Macherey-Nagel, Düren, Germany). Pigments were separated using a linear gradient system consisting of eluent A (90% methanol / 10% 0.5 M ammonium acetate, vol/vol) and eluent B (90% methanol/10% ethyl acetate, vol/vol). The flow rate over the column was 1.0 ml min^{-1}.

The individual peaks of the HPLC diagram were identified based on the respective absorption spectra and retention

times. Pigments were calculated in pmol using specific calibration factors and the DES (Dt/(Dt+Dd)) of the Dd cycle was calculated.

The DES of Ax/Vx and not of (Ax+Zx)/(Vx+Ax+Zx) was measured under HL for the green algae, as Zx could not be distinguished from lutein in the absorption spectrum of the chromatogram. Ax/Vx was calculated according to the area of each pigment in the chromatogram, without using a conversion factor. The DES of (Ax+Zx)/(Vx+Ax+Zx) was indirectly calculated as (Vx dark control - Vx light)/Vx dark control = 1 - (Vx light)/(Vx dark control), as the control value of Vx in the dark roughly corresponds to the total quantity of Vx+Ax+Zx in the light, assuming there is no *de novo* Vx synthesis during the 10 min of light stress exposure and most Ax and Zx are epoxidised to Vx in dark acclimation conditions. Vx-light is the control value of Vx or the Vx-value after insecticide treatment, both after 10 min of light exposure to 500 µE m^{-2} s^{-1}.

Table 4. Net oxygen evolution rate inhibition of the tested phytoplankton species under 150 µE m^{-2} s^{-1} light intensity, in presence of dimethoate and DCMU (positive control). 3 technical replicates (t.r.) for each dimethoate-treated sample , 1-3 t.r. for each DCMU-treated sample

Net O2 evolution rate inhibition [%]	C. reinhardtii	D. tertiolecta	Tetraselmis sp.	P. tricornutum	T. pseudonana
3 µmol dimethoate µg-1 Chl a	25 ± 6	53 ± 2	52 ± 1	58 ± 4	52 ± 3
45 pmol µg-1 Chl a DCMU	49 ± 10 (interpolated value)	-	79 ± 4	48 ± 5	71

Table 5. Respiration rate of *D. tertiolecta* and *Tetraselmis* sp. measured in the dark, in presence of increasing dimethoate concentrations

Respiration rate [µmol mg^{-1} Chl a h^{-1}]	control	3 µmol dimethoate $µg^{-1}$ Chl a (3. t.r.)	6 µmol dimethoate $µg^{-1}$ Chl a (1 t.r.)	18 µmol dimethoate $µg^{-1}$ Chl a (1 t.r.)
D. tertiolecta	53 ± 2 (3 t.r.)	63 ± 3	39	30
Tetraselmis sp	104 ± 15 (2 t.r.)	96 ± 12	57	39

3. Results and discussion

3.1. Oxygen evolution measurements

Absolute net oxygen evolution rates for the tested species (Table S1) are in a comparable range as usually reported for these species (Geel *et al.*, 1997; Ruffle *et al.*, 2001; Bailleul *et al.*, 2010; Chen *et al.*, 2021). Net oxygen evolution rate inhibition of the tested algae in presence of 3 µmol dimethoate $µg^{-1}$ Chl a is about 50-60%, except for *C. reinhardtii* with 25% (Table 4 and Table S1). Apparently, the last species is less sensitive to dimethoate than the other tested species. 45 pmol DCMU $µg^{-1}$ Chl a inhibits the oxygen evolution rate of the selected species similar or stronger compared to the mentioned dimethoate concentration, although the DCMU concentration was several orders of magnitude lower than dimethoate (Table 4).

The lowest observed effect concentration (LOEC) of dimethoate is 0.125 µmol $µg^{-1}$ Chl a for *D. tertiolecta* and *Tetraselmis* sp., 0.250 µmol $µg^{-1}$ Chl a for *T. pseudonana*, 0.5 µmol $µg^{-1}$ Chl a for *P. tricornutum* and 2 µmol $µg^{-1}$ Chl a for *C. reinhardtii*. Mohapatra *et al.* (1997) reported a significant effect of dimethoate on net photosynthetic oxygen production and photosynthetic carbon fixation of *Synechocystis* sp. PCC 6803 at concentrations higher than 50 µM.

Dimethoate does not remarkably affect the respiration of the tested phytoplankton species, except of *D. tertiolecta* and *Tetraselmis* sp., decreasing the respiration at concentrations ≥ 6 µmol dimethoate $µg^{-1}$ Chl a (Table 5). In similar studies it has been reported that low concentrations of dimethoate increased the respiration of the tested phytoplankton species. Piska and Waghary (1991) observed that about 50 µM dimethoate increased the respiration of the primary producers of a lake ecosystem and decreased the net photosynthetic production. Respiration was also found to be increased in *Anabaena* (Perona *et al.*, 1991b), *Nostoc* (Chen *et al.*, 2007) and *Synechocystis* (Mohapatra *et al.*, 1997) cells after adding dimethoate concentrations higher than 100 µM.

3.2. Chlorophyll fluorescence measurements

3.2.1. OJIP measurements

OJIP fluorescence measurement of *Tetraselmis* sp. (Figure 1a) depicts a representative trace of all OJIP measurements of the selected phytoplankton species in presence of dimethoate. The results show an increasing PSII inhibition with increasing dimethoate concentration (Figure 1a). However, even high concentrations like 18 µmol dimethoate $µg^{-1}$ Chl a do not inhibit PSII completely.

Dimethoate is acting like DCMU in all five tested phytoplankton species, disrupting the electron transport at the side of PSII beyond the primary acceptor (Q_A), as the O-J rise is related to the reduction of Q_A in PSII (Boisvert *et al.*, 2006). These findings are in agreement with Sridevi *et al.* (2012), who examined the effect of dimethoate as a.i. of Rogor on photosynthetic pigment fluorescence of *Chlorella vulgaris*. In our study, 45 pmol DCMU $µg^{-1}$ Chl a shows a similar PSII inhibition like 18 µmol dimethoate $µg^{-1}$ Chl a in *Tetraselmis* sp. (Figure 1b), and like 3 µmol dimethoate $µg^{-1}$ Chl a in the other four tested phytoplankton species. Clearly, dimethoate does not act like DBMIB (Figure 1b), which binds at the cyt b_6f complex at low concentrations (0.5 nmol DBMIB $µg^{-1}$ Chl a), which is manifested by a maximum of fluorescence at the I-level and with no distinguishable I-P transition (Lepetit & Dietzel, 2015). Mohapatra *et al.* (1996) demonstrated that dimethoate affects the PSII activity and phosphorylation of *Synechocystis* at all tested concentrations (10-3000 µM). In contrast to the studies showing that dimethoate inhibits

PSII, Chen *et al.* (2007) reported that dimethoate inhibits the ETC between PSII and PSI or even the dark reaction of the Cyanophyceaen species *Nostoc*. According to this author, dimethoate (2 mM) significantly increases the electron transport activity from water to methylviologen by 193% compared to the control and the PSI activity by more than 400% compared to non-treated cultures. Our data indicate a binding of dimethoate at the PSII core, partially preventing the electron transfer from Q_A towards the cyt b_6f complex.

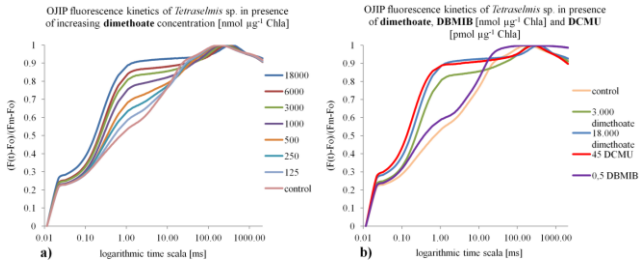


Figure 1. OJIP fluorescence kinetics of *Tetraselmis* sp. after double normalisation in presence of **a)** increasing dimethoate concentrations [nmol µg^{-1} Chl a] and **b)** dimethoate compared to DBMIB [nmol µg^{-1} Chl a] and DCMU [pmol µg^{-1} Chl a]. Dimethoate inhibits the PSII beyond Q_A like DCMU (positive control), while DBMIB (positive control) the cyt b_6f complex. F_v: variable fluorescence; F_o: minimum fluorescence; F_m: maximum fluorescence. Two technical replicates (t.r.) were taken per reagent concentration

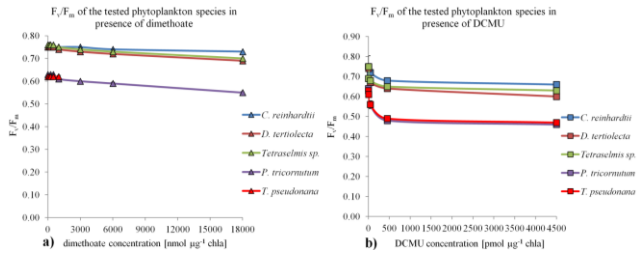


Figure 2. Effect of increasing **a)** dimethoate and **b)** DCMU concentrations on maximum photochemical efficiency (F_v/F_m) of the five selected phytoplankton species. Two technical replicates (t.r.) per F_v/F_m value

The effect of dimethoate (Figure 2a) and DCMU (Figure 2b) on maximum photochemical efficiency (F_v/F_m) of the tested phytoplankton species is similar. The three green algal species show similar F_v/F_m values under different dimethoate concentrations and remarkable higher values than the two diatoms. The control values of F_v/F_m for the used green algae and diatoms are 0.74-0.75 and 0.64, respectively (Table 7), which is in accordance with the literature (Hofstraat *et al.*, 1994; Bonenta *et al.*, 2012; Lepetit *et al.*, 2017; Qin *et al.*, 2021). In the five phytoplankton species, F_v/F_m is slightly decreasing with increasing dimethoate concentrations, and clearly different from the control at concentrations higher than 3-6 µmol dimethoate µg^{-1} Chl a, depending on the species. Apparently, dimethoate does not decouple antennas from PSII. Otherwise, the free moving antennas would fluoresce stronger in the ground fluorescence (higher F_o), resulting in a much lower F_v/F_m compared to the control.

3.2.2. NPQ measurements

As outlined before, NPQ is a complex phenomenon that relies on different mechanisms, classically qE, qT and qI, though recently other factors have been added, such as qH (Malnoe, 2018) and qZ (Nilkens *et al.*, 2010). Thus, NPQ analyses alone do not provide major insights into the respective mechanisms. However, as on a short-term scale NPQ processes are usually triggered directly or indirectly by the delta pH (which is also connected to the redox state of the plastoquinone pool, the trigger for state transitions), we performed NPQ analyses to further corroborate the effect of dimethoate on the photosynthetic ETC. NPQ in the light (NPQ_L) decreases with increasing dimethoate concentrations in the five tested phytoplankton species compared to the control (Figure 3a-e), probably because dimethoate inhibited PSII and thus the establishment of a transthylakoidal proton gradient, the prerequisite for qE. NPQ_L development of *P. tricornutum* is similar to *Tetraselmis* sp. in presence of dimethoate (Figure 3d,c). 3 µmol dimethoate µg^{-1} Chl a showed a similar effect on NPQ_L development as 45 pmol DCMU µg^{-1} Chl a in *C. reinhardtii*, *Tetraselmis* sp. and *T. pseudonana* (Figure 3a,c,e).

NPQ_L measured in *C. reinhardtii* was low, as the LhcSR pigment-binding proteins that induce NPQ_L are little expressed under the tested light conditions. According to Nawrocki *et al.* (2020), the expression of these complexes depends on prior HL exposure of the cells. A negative development of NPQ_L was observed in presence of the photosynthetic inhibitors over time, potentially induced by state transition, which is very prominent in *C. reinhardtii* (Nawrocki *et al.*, 2016).

In *D. tertiolecta*, NPQ_L increases over time (Figure 3b). In the presence of dimethoate, this increase is lowered as expected, with high concentrations of dimethoate completely suppressing NPQ development.

NPQ_L of *T. pseudonana* steeply increases during the first 50 seconds and strongly decreases afterwards at concentrations up to 1 µmol dimethoate µg^{-1} Chl a (Figure 3e). Probably, the immediate increase of NPQ is directly triggered by the proton gradient alone and is relatively independent of the xanthophyll cycle. The Calvin cycle needs some time to get activated and thus the proton gradient is especially high directly after light onset. After the activation of the Calvin cycle, NADPH and ATP are consumed, resulting in a partial proton gradient relaxation, followed by an NPQ_L decrease. A similar NPQ kinetic has been observed in the highly related diatom *Cyclotella meneghiniana*, where such a fast, pH dependent qE component was characterized in depth (Grouneva *et al.*, 2008, 2009)

NPQ in the dark (NPQ_{DR}) of the phytoplankton species treated with 3 µmol dimethoate µg^{-1} Chl a is similar to the control and to the 45 pmol DCMU µg^{-1} Chl a-treated species (Figure 4), which indicate that no photoinhibition of the algae occurs after pesticide application.

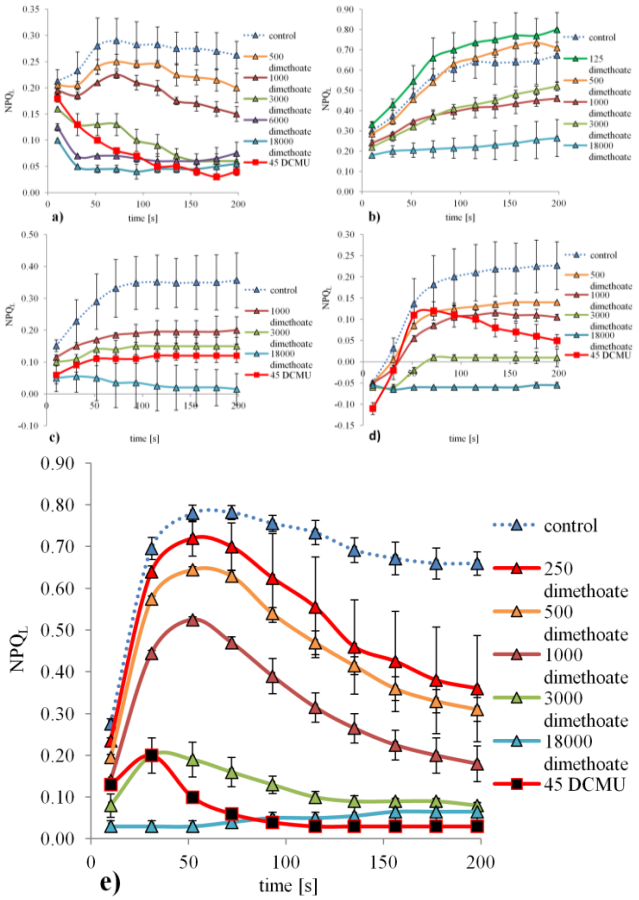


Figure 3. Effect of increasing dimethoate concentrations [nmol µg^{-1} Chl a] on NPQ development in the light (NPQ_L) of **a)** *C. reinhardtii*, **b)** *D. tertiolecta*, **c)** *Tetraselmis* sp., **d)** *P. tricornutum* and **e)** *T. pseudonana*. DCMU: positive control. Two technical replicates were taken for each NPQ_L value

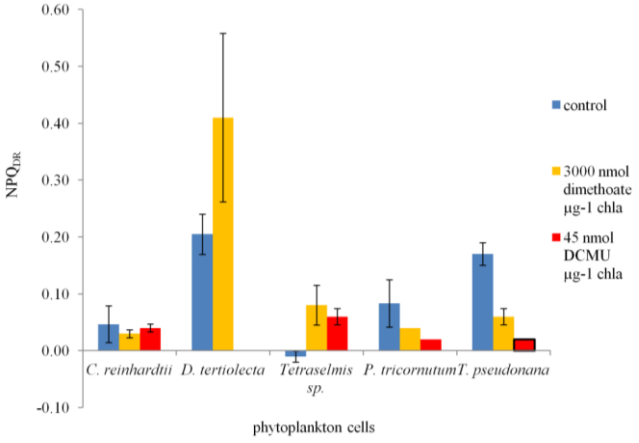


Figure 4. Effect of dimethoate and DCMU on NPQ dark relaxation (NPQ_{DR}) of the tested phytoplankton species. DCMU: positive control. 2-3 technical replicates (t.r.) per control, 2 t.r. per reagent-treated sample, except of one t.r. for DCMU-treated *T. pseudonana* (black edging). No NPQ_{DR} value exists for DCMU-treated *D. tertiolecta*

3.3. Xanthophyll pigment measurements

Another piece of evidence for the acting site of dimethoate in the photosynthetic apparatus was obtained through xanthophyll pigment analysis by HPLC. While we could measure the de-epoxidation state Dt/(Dd+Dt) for the diatoms, in the green algae we could not distinguish Zx from lutein in our HPLC approach. Hence, we measured Ax/Vx, although Ax/Vx alone does only provide limited information about the operating xanthophyll cycle, as a low Ax/Vx ratio could indicate a general low xanthophyll cycle activity, but also hide a fast de-epoxidation of Ax into Zx. However, the DES (Ax+Zx)/(Vx+Ax+Zx) can be converted into 1 - (Vx light)/(Vx dark control) and here the relative decrease of Vx reflects the xanthophyll cycle activity (for details see chapter 2.6). The xanthophyll DES measurements show that dimethoate acts like DCMU. Due to its potential binding at the Q_B site of PSII, dimethoate disrupts the electron flow towards the cytb_6f complex, and thus decreases the proton gradient, that is responsible for activating the de-epoxidation reaction. Ax/Vx of the tested green algal species (Figure 5a) and Dt/(Dd+Dt) of the diatom species (Figure 5c) decreased compared to the control in presence of dimethoate and DCMU, while a Vx de-epoxidation was obvious under HL conditions, in absence of the pesticides.

As expected, 1 - (Vx light)/(Vx dark control) decreased under HL conditions in dimethoate and DCMU-treated *C. reinhardtii* and *Tetraselmis* sp. cells compared to the control (Figure 5b). Interestingly, this parameter even reached negative values in *D. tertiolecta*. Probably, the HL exposure time was too low in order to start a pronounced de-epoxidation in this species, in line with results obtained by Casper-Lindley and Björkman (1998), and at the same time, there is some de novo synthesis of Vx.

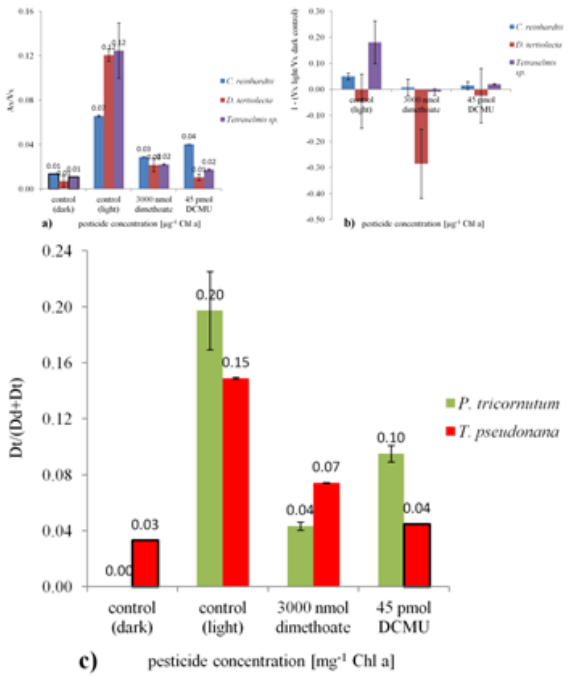


Figure 5. Effect of dimethoate and DCMU under HL conditions on the de-epoxidation state of **a)** Ax/Vx and **b)** 1 - (Vx light/Vx dark control) that corresponds to (Ax+Zx)/(Vx+Ax+Zx) of the 3 tested green algal species and **c)** Dt/(Dd+Dt) of the 2 diatom species. Vx light: Vx value of the control or the insecticide-treated sample under HL conditions; Vx dark control: control value of Vx in the dark. DCMU: positive control. At least 2 technical replicates (t.r.) for each measurement; columns with black edging correspond to one t.r.

3.4. Effect of Perfekthion on photosynthesis of the five selected phytoplankton species

As with dimethoate and DCMU, the net oxygen evolution rate inhibition increases with increasing concentrations of Perfekthion in the five phytoplankton species. A representative trace (R.T.) for all net oxygen evolution measurements is depicted for *P. tricornutum* (Figure 6a). The single net oxygen evolution rate data of the pesticide-treated *P. tricornutum* is listed in Table S2 (supplement). The LOEC of the tested phytoplankton cells is 0.05 µmol Perfekthion µg^{-1} Chl a. 0.5 µmol Perfekthion µg^{-1} Chl a inhibits the net oxygen evolution rate of the tested phytoplankton species by more than 90%, while the same concentration of dimethoate shows an inhibition of 14-35% (the value of *C. reinhardtii* was not remarkably different to the control). Perfekthion inhibits the photosynthesis of the tested phytoplankton species stronger than dimethoate, probably because of an additional effect of the solvents. Panda *et al.* (1998) observed that concentrations of Perfekthion (there named Rogor) higher than 100 µM increase the permeability of the plasma membrane in *Chlorella vulgaris*.

Perfekthion does not affect the respiration of the tested phytoplankton species, except of *Tetraselmis* sp., where the respiration is decreased in presence of a higher concentration, i.e. 2 µmol Perfekthion µg^{-1} Chl a (Table 6). This high concentration was not tested in the other two green algae.

Table 6. Respiration rate of *Tetraselmis* sp. measured in the dark, in presence of Perfekthion. t.r.: technical replicate.

	Control (3 t.r.)	**0.5 µmol Perfekthion µg-1 Chl a (3 t.r.)**	**2 µmol Perfekthion µg-1 Chl a (2 t.r.)**
Respiration rate [µmol mg^{-1} Chl a h^{-1}]	60 ± 18	57 ± 9	5 ± 6

Table 7. F_v/F_m of the tested phytoplankton species after addition of dimethoate and Perfekthion. b.r./t.r.: biological/technical replicate. Remarkable F_v/F_m-values in bold font.

Insecticide concentrations [µmol µg-1 Chl a]	**C. reinhardtii**	**D. tertiolecta**	**Tetraselmis sp.**	**P. tricornutum**	**T. pseudonana**
control(2-3 b.r.)	0.75 ± 0.00	0.75 ± 0.01	0.74 ± 0.04	0.64 ± 0.01	0.64 ± 0.02
18 dimethoate(2 t.r.)	0.73 ± 0.001	0.69 ± 0.01	0.70 ± 0.002	0.55 ± 0.005	0.59 (1 t.r.)
0.5 Perfekthion(2 t.r.)	0.51 ± 0.01	0.31 ± 0.06	0.48 ± 0.05	0.58 ± 0.03	0.38 ± 0.01
1.0 Perfekthion(2 t.r.)	0.30 ± 0.02	0.16 ± 0.02	0.40 ± 0.04	0.34 ± 0.12	0.34 ± 0.01

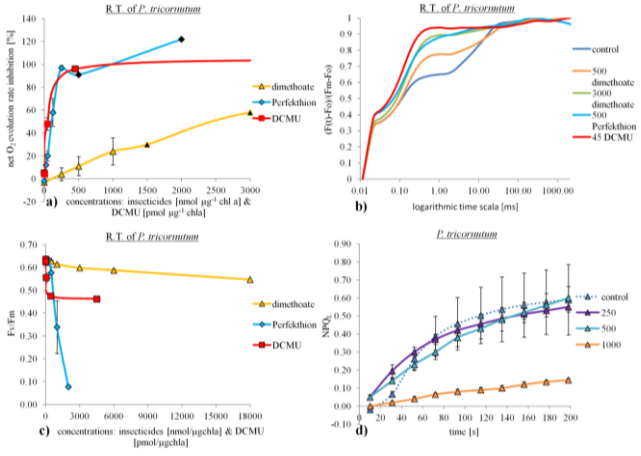


Figure 6. Effects of increasing concentrations of dimethoate and Perfekthion [nmol µg^{-1} Chl a] and DCMU [pmol µg^{-1} Chl a] on **a)** net oxygen evolution rate (b.r.), **b)** OJIP fluorescence (2 technical replicates (t.r.)), **c)** F_v/F_m (t.r.) and **d)** NPQ_L development (t.r.) of *P. tricornutum*. DCMU: positive control. Black filled symbols are mean values of 1 t.r./b.r., black framed symbols are mean values of 2 t.r./b.r. and symbols with no black colour at all are mean values of 3 t.r./b.r.. F_v: variable fluorescence; F_o: minimum fluorescence; F_m: maximum fluorescence; R.T.: representative trace; t.r/b.r.: technical/biological replicate.

According to the OJIP fluorescence measurements, 0.5 µmol Perfekthion µg^{-1} Chl a inhibits PSII of the tested algae similar to 3 µmol dimethoate µg^{-1} Chl a and 45 pmol DCMU µg^{-1} Chl a (Figure 6b). But 0.5-1.0 µmol Perfekthion µg^{-1} Chl a decreases F_v/F_m of the used phytoplankton species even stronger than 18 µmol dimethoate µg^{-1} Chl a (Table 7 and Figure 6c). At these concentrations, the solvents of Perfekthion probably make the cell membranes highly permeable to the active ingredient (dimethoate), and dissociate either the chlorophylls from the proteins or the light harvesting complex from PSII, which leads to an increased F_o fluorescence. The traces for *P. tricornutum* (Figure 6b,c) are representative for the respective experiments.

The effect of Perfekthion on NPQ_L of *P. tricornutum* (Figure 6d) was similar to the effect of dimethoate (Figure 3d). Concentrations higher than 1 µmol Perfekthion µg^{-1} Chl a suppressed NPQ_L development.

0.5 µmol Perfekthion µg^{-1} Chl a increases NPQ_{DR} in *C. reinhardtii* and *P. tricornutum* to values 5-10 times higher than the control or 3 µmol dimethoate µg^{-1} Chl a (Figure 7), indicating a photoinhibition in the first species and potentially a Dt-dependent quenching (qZ) with a combination of qI in *P. tricornutum*. The photosystems get probably destroyed due to the block of the photochemical quenching channel, resulting in increased triplet chlorophyll species and eventually higher amounts of singlet oxygen. Additionally, Perfekthion may even detach chlorophylls from the pigment binding proteins. However, according to Sridevi (2012), Rogor does not dissociate the LHC in *C. vulgaris* cells (up to 500 µM tested), but the organophosphate insecticides quinalphos and chlorfenvinphos dissociate it. The same author reported that the electron flow between the oxygen-evolving-complex (OEC) and PSII is not significantly inhibited in presence of Rogor. To make a clear statement about

photoinhibition longer NPQ_{DR} measurements would be necessary.

While the xanthophyll DES measurement of the tested phytoplankton species show that dimethoate acts like DCMU, in *P. tricornutum*, DCMU shows a high de-epoxidation (Figure 8), well known from other studies (Grouneva *et al.*, 2009; Lepetit *et al.*, 2013). It is possible that the complete inhibition of PSII by DCMU, in contrast to the partial inhibition by dimethoate, leads to additional physiological processes, such as a strong PSI cyclic electron transport that increases the transthylakoidal proton gradient. Future experiments could show if the increase of Dt/(Dd+Dt) in presence of Perfekthion is attributed to the same effect as imposed by DCMU, and whether a probable destruction of the photosystems would be the reason that this effect gets reversed by higher insecticide concentrations.

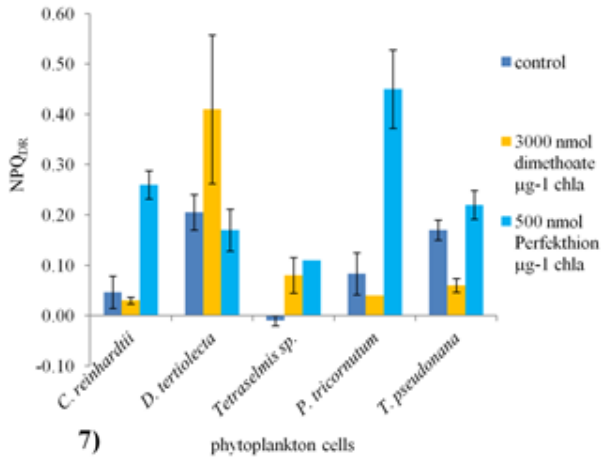


Figure 7. Effect of Perfekthion on NPQ_{DR} of the tested phytoplankton species compared to the control and dimethoate treatment. Two technical replicates were taken for each measurement.

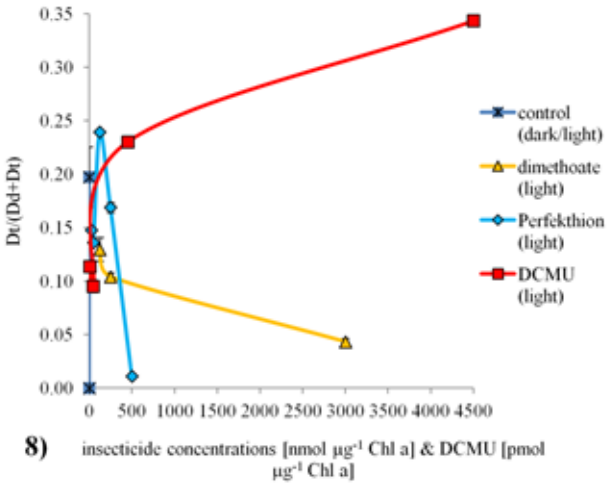


Figure 8. Effect of increasing dimethoate, Perfekthion and DCMU concentrations on the DES Dt/(Dd+Dt) of *P. tricornutum*. DCMU: positive control. Black filled symbols: mean values of 1 t.r.; black framed symbols: mean values of 2 t.r.; symbols with another colour than black: mean values of 3 t.r.

Perfekthion contains 37.2% dimethoate (372 g l^{-1} or 1.62 M). According to Raiffeisen (online) and Profiflor GmbH (online), BVL and the chemical producing company BASF (BASF SE, Ludwigshafen, Germany) respectively, recommended a dilution of Perfekthion in water of 0.83/1000-3.5/1000, before applying on plants on the field. Thus, the concentration to be sprayed on the field should be 1.3-5.7 mM, depending on the kind and size of the plant, and the pest. The LOECs of Perfekthion measured on net oxygen evolution rate of the tested phytoplankton species was 50 nmol μg^{-1} Chl a (100 µM), demonstrating that the applied field concentration is ca. 10-60 times higher than the LOEC. The sprayed volume of diluted Perfekthion per hectare should be 12-600 l (Raiffeisen, Profiflor, online). If the water volume after a rainfall is estimated to be 10-50 l m^{-2}, the final concentration of Perfekthion on the field after a rainfall would be 0.03-34.2 µM. After a dry period, evaporation could enhance the concentration of dimethoate in the commercial formulation. On the other hand, dilution of Perfekthion in the aquatic environment is expected to decrease its concentration to several orders of magnitude lower than applied on the field. In addition to the low persistence (few days) of Perfekthion in the environment (Petsas *et al.*, 2007), it is unlikely that Perfekthion affects the photosynthesis of the tested algae in the aquatic environment.

4. Conclusions

Net oxygen evolution rate inhibition of the tested phytoplankton species increases with increasing dimethoate concentrations. Dimethoate does not affect the respiration of the algae, except of decreasing the respiration of *D. tertiolecta* and *Tetraselmis* sp. at concentrations higher than 6 µmol μg^{-1} Chl a. OJIP fluorescence measurements show that dimethoate inhibits the acceptor side of PSII beyond Q_A, similarly to DCMU, and does not inhibit PSI. In presence of 3 µmol dimethoate μg^{-1} Chl a, F_v/F_m and NPQ_{DR} are similar to the control and thus, no indication of photoinhibition was observed. Increasing dimethoate concentrations decreased the xanthophyll DES Ax/Vx and Dt/(Dd+Dt) and decreased NPQ_L, an indication of a blocked ETC. In all the experiments 3 µmol dimethoate μg^{-1} Chl a act comparable to 45 pmol DCMU μg^{-1} Chl a.

Perfekthion, the commercial formulation of dimethoate, shows a stronger inhibition on the net oxygen evolution rate and on PSII than dimethoate. 2 µmol Perfekthion μg^{-1} Chl a decreased the respiration of *Tetraselmis* sp.. 0.5-1 µmol Perfekthion μg^{-1} Chl a decreased F_v/F_m, increased NPQ_L development and increased NPQ_{DR} in most of the phytoplankton species compared to 3 µmol dimethoate μg^{-1} Chl a, indicating that probably the solvents of Perfekthion dissociated the pigments and/or LHCs from PSII.

According to the study, if the recommended concentration of Perfekthion on the field is used, it is unlikely that it affects the photosynthesis of the selected phytoplankton species in the aquatic environment after a rainfall. More specialised studies will help to identify the exact inhibiting location of the insecticide beyond Q_A. Future research will be necessary to examine possible effects of Perfekthion on the oxygen-evolving complex, the LHC, the mitochondria

and the membrane permeability of the phytoplankton cells.

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Supplementary information

Table S1. Net oxygen evolution rates [µmol mg^{-1} Chl a h^{-1}] of each tested phytoplankton species after treatment with dimethoate and DCMU. 3 technical replicates (t.r.) per measurement, unless otherwise stated. Control 1 and control 2 were measured on different days.

	C. reinhardtii	**D. tertiolecta**	**Tetraselmis sp.**	**P. tricornutum**	**T. pseudonana**
Control 1	119 ± 33	189 ± 17	303 ± 13(2 t.r.)	193 ± 12	164 ± 44
3 µmol dimethoate $µg^{-1}$ Chl a	93 ± 15	85 ± 8	146 ± 10	77 ± 8	92 ± 9
Control 2	120 ± 13	-	209 ± 5	156 ± 27	204(1 t.r.)
45 pmol DCMU $µg^{-1}$ Chl a	60(interpolated value)	-	44 ± 11	84 ± 10	63(1 t.r.)

Table S2. Net oxygen evolution rates [µmol mg^{-1} Chl a h^{-1}] of *P. tricornutum* after treatment with dimethoate, Perfekthion and DCMU. 3 technical replicates (t.r.) per measurement, unless otherwise stated. b.r. = biological replicate. Control 1, 2, 3 were measured on different days.

Dimethoate concentrations [µmol $µg^{-1}$ Chl a]	**1 b.r.**	**Perfekthion concentrations [µmol $µg^{-1}$ Chl a]**	**2 b.r. unless otherwise stated**	**DCMU concentrations [pmol $µg^{-1}$ Chl a]**	**1 b.r.**
0 (control 1)	193 ± 12	0 (control 3)	162 ± 4	0 (control 2)	156 ± 27
0.25	170 ± 2	0.025	115 ± 18	4.5	162 ± 4 (2 t.r.)
0.5	145 ± 3	0.05	106 ± 4	45	84 ± 10
1.0	123 ± 5	0.125	60 ± 12	450	7 ± 2 (2 t.r.)
1.5	91 ± 5	0.25	6 ± 5	4500	-8 ± 11 (2 t.r.)
3.0	77 ± 8	0.5	15 ± 21 (1 b.r., 2 t.r.)		
		2.0	-32 ± 19 (1 b.r., 2 t.r.)		